Pergamon

Oral Oncol, Eur J Cancer, Vol. 31B, No. 4, pp. 267–274, 1995
Elsevier Science Ltd
Printed in Great Britain
0964–1955/95 $9.50+0.00

0964-1955(95)00020-8

The Effect on Cell Phenotype of the Mutagenic Peptide of Herpes Simplex Virus Type-1

C.M. Das, V.A. Murrah, S. Zhang, G. Wong, E.P. Gilchrist and E.J. Shillitoe

The transforming region of the genome of herpes simplex virus type-1 (HSV-1) encodes a peptide that raises the mutation frequency of cells. To find the effect of this peptide on cell phenotype, three types of cells were transfected with a shuttle vector plasmid that expressed the peptide. When immortalised rat fibroblasts were transfected they rapidly became anchorage-independent with high efficiency, but were not tumorigenic in nude mice. When monkey kidney cells were transfected, five clonal cell lines were isolated, of which one became anchorage-independent but was not tumorigenic in nude mice. When human oral keratinocytes were transfected they did not become immortalised. The peptide therefore induced some of the features of transformation in different cell types, but did not induce a malignant phenotype in any cell. This suggests that interaction with co-factors would be necessary for the peptide to contribute to the development of oral cancer.

Keywords: herpes simplex, transformation, cancer

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INTRODUCTION

THE ORAL mucosa is regularly exposed to agents which are mutagenic or carcinogenic under experimental conditions. These include herpes simplex virus type-1 (HSV-1), human papillomaviruses, and components of tobacco smoke [1]. Although these agents can transform cells in culture, the mechanisms by which each one operates is not fully understood. This makes it difficult to analyse a tumour and decide which, if any, particular agent was responsible for its induction. In particular, the relationship between the herpes simplex virus and oral cancer is obscure, and rests on studies of antibodies and tissues performed over a decade ago [2, 3].

It is known that HSV-1 can transform cells into a malignant phenotype, using a 'hit-and-run' mechanism in which viral DNA is not retained by the transformed cells [4]. Transformation does not require the entire viral genome, but can be accomplished by a region known as *mtr*-1. Within *mtr*-1 we have located an open reading frame within the *UL26* gene which encodes a peptide that is expressed in virus-infected cells [5]. The peptide raises the mutation frequency of cells [6, 7] and sequence analysis shows that it has some characteristics of the transforming proteins of other tumour viruses [1]. The peptide is therefore a candidate for a transforming protein of HSV-1. The present study was performed to find if this peptide could indeed transform cells into a malignant phenotype, with the eventual goal of using the peptide in later studies of human oral cancers.

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Received 19 Dec. 1994; provisionally accepted 20 Feb. 1995; revised manuscript received 16 Mar. 1995.

MATERIALS AND METHODS

Cells

Immortalised rat fibroblasts (NRK536 cells) were kindly provided by Dr U. Rapp (NIH, Bethesda, Maryland, U.S.A.). They were maintained in Eagle's minimum essential medium containing 5% fetal bovine serum (FBS) supplemented with 100 U/ml penicillin, 100 U/ml streptomycin, 0.25 μg/ml amphotericin B and 280 μg/ml glutamine.

Vero cells, Cos-1 cells, HeLa cells and SV-T2 cells were obtained from the American Type Culture Collection (Rockville, Maryland, U.S.A.). They were maintained in M199 containing 10% FBS, penicillin, streptomycin and amphotericin B. Transfected Vero cells were maintained in the same medium containing 400 μg/ml G418.

Primary human oral keratinocytes were obtained from biopsy specimens obtained from the Department of Oral and Maxillofacial Surgery, University of Texas Health Science Center, San Antonio, Texas, U.S.A., or from cadaveric donors at autopsy. Tissues were processed by dissecting away the connective tissue and dicing the epithelium into 0.5–1.0 mm cubes. Tissue pieces were rinsed in minimum essential

medium with antibiotics and incubated for 10 min in sterile RBC-lysing buffer. They were again rinsed and cultured under conditions to suppress fibroblasts while encouraging epithelial cell growth [8–10].

Plasmids

A 378 base pair region of the transforming region of the HSV-1 genome was removed from the plasmid pUC-9-G-B [6] and cloned in both orientations into the *Eco* RV site of the expression vector pMAM*neo*-BLUE (Clontech Laboratories Inc, Palo Alto, California, U.S.A.). The cloned fragment consisted of the 5′ end (in the prototype orientation) of the *Bam* HI fragment G of the genome of HSV-1 and included the 3′ end of the *UL26* open reading frame. The fragment was cloned adjacent to the MMTV promoter, and a eukaryotic start codon was included in the construct. pDB337 contains the viral sequences in the correct orientation relative to the promoter and pDB339 has the viral sequences in the opposite orientation. The constructs were described previously [7]. The viral peptide that can be expressed from pDB337 has the amino acid sequence IPTGGIQRGGRGPCQRQQRSTR-GR.

Synthetic peptide and antisera

The synthetic peptide was synthesized according to the sequence shown above and rabbit and goat antisera were obtained as described previously [5]. Pre-immune sera from the same animals were used as controls.

Transfection

NRK536 cells were transfected when the cells were between passage 10 and 30. Cells were plated in 100 mm^2 dishes and after 24 h, while still sub-confluent, they were either transfected by calcium phosphate precipitation with plasmid DNA that had been purified on a Qiagen column (Qiagen Inc., Chatsworth, California, U.S.A.) [11] or were infected with herpes simplex virus that had been exposed to UV light as previously described [7]. After 48 h the cells were harvested and 2×10^4 cells were seeded directly into 0.3% Sea Plaque agarose (Marine Biocolloids, Rockland, Maine, U.S.A.) in complete medium above a bottom layer of 0.5% agarose in 6-well tissue culture plates. Cultures were maintained at 37°C and colonies were counted after 2 weeks.

Vero cells were transfected with CsCl gradient-purified plasmids by electroporation with a Bio-Rad Gene Pulser (Bio-Rad Laboratories, Richmond, California, U.S.A.). For each experiment 3×10^6 Vero cells and 100 μg plasmid DNA were used and electroporation was performed at 200 V and 500 μF capacitance. The electroporated cells containing plasmid DNA were selected for neomycin resistance in M199 with 10% FBS and G418 at a final concentration of 400 μg/ml. Fresh G418-containing medium was added every 2–3 days until G418-resistant colonies of over 500 cells were apparent at around 10–12 days. Transfection efficiencies were calculated to be 7×10^{-6}, 5×10^{-6}, and 3×10^{-6} for the plasmids pMAM*neo*-BLUE, pDB337 and pDB339, respectively. Colonies were trypsinised, picked and subcultured in 75 mm^2 flasks. From a viable transfected cell culture a series of limiting dilutions were made in the same media and plated into wells of 96-well plates with an average of less than one cell per well and a set of clonal cell lines was obtained by transferring them to flasks containing the same medium with 800 μg/ml G418 and 1% FBS.

Human oral keratinocyte cultures were transfected with Lipofectin reagent (BRL, Gaithersburg, Maryland, U.S.A.) [12]. For each culture well, 10 μg of appropriate plasmid DNA in 500 μl of serum and antibiotic-free keratinocyte defined medium (KDM) [9] was mixed with 500 μl KDM containing 15 μg of Lipofectin reagent and allowed to incubate for 15 min. Cultures were rinsed three times with KDM to remove residual serum, after which the DNA/Lipofectin solutions were placed on the appropriate cultures. Control cultures were untreated or vehicle-treated with 15 μg of Lipofectin. After 12–16 h 1 ml of antibiotic-free KDM containing 1% fetal bovine serum was added to each culture. After an additional 24 h the cultures were rinsed three times with complete KDM and then re-fed with 1 ml of complete KDM containing 5 μM dexamethasone to induce expression of the peptide. The cells were observed daily and fed weekly. When cultures were confluent they were split into two flasks. This continued until the cultures became senescent and were lost.

Confirmation of transfection

Each cell line derived from Vero cells was tested repeatedly to ensure that it carried the appropriate plasmid. Cells that carried pDB337 were further tested to ensure that the peptide was expressed. Three different techniques were used.

Immunohistochemical staining. Cell lines were examined for expression of the HSV-1 peptide by an indirect immunoperoxidase technique as previously described [13]. Individual lines were subcultured and 10^4 cells plated on both chambers of a LabTek slide and one chamber induced for 24 h with 1 μM or 25 μM dexamethasone to assay for expression of peptide. Indirect immunoperoxidase staining was carried out with an ABC stain (Vector Laboratories, Burlingame, California, U.S.A.) following preincubation with 0.5% H_2O_2 in methanol for 30 min at room temperature to quench endogenous peroxidase activity. Cells were examined by two observers, under code. Clonal lines were scored positive if they showed darker brown staining in 100% of the cells relative to the cell lines transfected with control plasmids PMAM*neo*-BLUE or pDB339 and untransfected Vero cells reacted under the same conditions. The lines were examined regularly at passages up to 15, at which time all other assays had been completed.

To find the transfection efficiency of the human keratinocytes, parallel cultures were transfected with the plasmid pZ189 [14]. After 3 days the immunoperoxidase staining technique was performed with a monoclonal antibody to the SV40 T antigen (Oncogene Science Inc., Uniondale, New York, U.S.A.), and stained cells were counted in randomly-selected fields. As a positive control, SV-T2 cells were also stained, since all of these cells express the T antigen.

Enzyme-linked immunosorbent assay (ELISA). The ELISA assay was also used to confirm expression of the peptide [5, 15]. Cells were grown to confluent monolayers in the presence or absence of dexamethasone for 2 days in 150 mm dishes and were harvested 24 h after the addition of fresh medium with or without dexamethasone. Briefly, the dishes were chilled to 0°C, rinsed twice with ice cold PBS with 0.001 M EDTA and 0.6 μM phenylmethylsulphonyl fluoride and removed with a

rubber scraper. Cells were recovered by centrifugation at 200 *g* for 5 min, washed once and finally suspended in 0.5 ml of the same buffer by vortexing vigorously in a microcentrifuge tube. The cells were disrupted by three cycles of freezing in dry ice and ethanol and thawing at 37°C, followed by centrifugation at 12 000 *g* for 5 min and the supernatant was collected in a fresh microcentrifuge tube and used as a source of antigen for the ELISA. The entire procedure for extract preparation was performed at 4°C. Protein concentrations were determined by the BCA protein assay (Pierce Chemical Co., Rockford, Illinois, U.S.A.) and 100 μg of the total proteins were used to coat the wells of a 96-well plate overnight at 4°C.

Polymerase chain reaction. To confirm that cells retained the appropriate plasmid, the polymerase chain reaction (PCR) was employed. Primers consisted of the M13 universal forward and reverse primers (USB, Cleveland, Ohio, U.S.A.) to anneal either side of the cloning site into which the HSV sequences had been inserted. DNA extracts of Vero cells, and pDB337 plasmid DNA acted as negative and positive controls, and the PCR was performed as previously described [16]. Reaction mixtures were electrophoresed on 2% agarose gels and bands of appropriate size were confirmed as viral DNA by hybridisation to a ^{32}P-labelled pDB337 DNA probe.

Growth rate

Growth rates were determined in newly expanded clones that expressed the peptide as well as two non-expressing lines that had been transfected with the vector, or with PDB 339. The cells (1×10^4) were plated into wells of a 24-well culture plate in the presence of 25 μM dexamethasone. At 24-h intervals, duplicate wells of each cell type were trypsinised, and the cells were counted in a haemocytometer chamber. Growth curves for individual cell lines were determined immediately after clonal expansion.

Anchorage independence

To determine if expression of the peptide induced anchorage-independent growth in Vero cells, 1×10^5 cells were pre-incubated in the presence of 25 μM dexamethasone for 2 days, then suspended in 1.5% methylcellulose with G418 but no dexamethasone. The suspension was plated on to 60 mm dishes coated with 0.8% sterile agarose and incubated at 37°C for 21 days. The dishes were then examined for colony forming ability on soft agar, and colonies having a diameter greater than 100 mm were counted [17]. HeLa cells were included as a positive control.

Serum dependence

To determine if the peptide increased the ability of Vero cells to form colonies in low serum, 500 cells were plated on to 100 mm dishes in M199/G418 medium containing either no serum, 0.2, 0.4, 0.6, 0.8, 1, or 10% serum. Expression of the peptide was induced by 25 μM dexamethasone. After 12 days, the dishes were examined and the number of colonies were counted [17].

Mutation frequency

The frequency of mutations in cell lines was determined with a shuttle vector system [14]. The mutagenesis shuttle vector pS189 was modified by removal of the region that codes for ampicillin and its replacement with a kanamycin-resistance gene cassette (Pharmacia Inc., Piscataway, New Jersey, U.S.A.). This modified shuttle vector was named pK189. The vector was introduced into cells by calcium phosphate precipitation and glycerol shock and allowed to replicate for 48 h. It was then recovered by alkaline lysis, followed by digestion with *Dpn* I to remove any non-replicated plasmids, and was introduced into *E. Coli* MBM 7070. Plasmids with a mutation in the *sup* F gene were recognised by a colour change and were confirmed to be mutants by recovery and transfer into another bacterial culture. The mutation frequency was expressed as the proportion of white colonies recovered.

In order to classify the types of mutation that had occurred, mutant plasmids were recovered from overnight bacterial cultures, digested with *Bam* HI and the fragments were resolved in an agarose gel. Each mutant was confirmed to be derived from pK189 by examination of fragment sizes and the type of mutation was assessed by examination of the numbers and sizes of the fragments. The mutation was classified as a point mutation if two fragments were obtained with sizes that were indistinguishable from 4.3 and 0.9 Kb. The mutation was considered to be a deletion if the total size of the fragments was less than 5.2 Kb and classified as an insertion if the total size of fragments was greater than 5.2 Kb.

Tumorigenicity

To test cell lines for the ability to make tumours, either 1×10^6 or 1×10^7 cells were injected subcutaneously into groups of six nude mice. As a positive control, some animals were injected with equal numbers of the known tumorigenic SV-T2 cells, and other animals were injected with non-transfected Vero cells. Animals were examined daily for up to 3 months, after which time they were sacrificed. The major organs were examined at autopsy for the presence of tumours, and the skin at the site of injection was examined histologically for any foci of tumours.

Statistical analysis

Mutation frequencies were compared by Fisher's exact test and the different proportions of different types of mutations were compared by a Chi square test.

RESULTS

Transformation of NRK536 cells

The spontaneous transformation frequency of NRK536 cells was low, with only two transformed colonies being observed per 2×10^4 cells, and the number was not increased by the addition of 10 mM dexamethasone. Transfection with pDB339 raised the number of colonies to five. The plasmid pDB337 produced 15 colonies in the absence of dexamethasone and 32 colonies when dexamethasone was added to the cultures.

HSV-1 that had been irradiated by UV light for 10 min led to 53 colonies, while irradiation of the virus for 15 min led to 31 colonies.

Transformation of Vero cells

A total of seven clonal cell lines were developed which grew continuously in medium with G418. One line was from Vero cells that had been transfected with the vector pMAM*neo*-BLUE alone, one was from Vero cells that had been transfected with pDB339, and the other five were from cells that had been transfected with pDB337. The cell lines that contained pDB337 were named H11.1, B7, B10, F7, and F9. The number of cells per 75 cm^2 flask ranged from 1×10^7 to

2×10^7 in these seven lines. Cell morphology was normal fibroblast-like and was very similar from clone to clone. However, the cells in the H11.1 line were much larger than the others.

Expression of viral peptide

To confirm expression of the viral peptide, all cell lines were examined by immunoperoxidase staining with antiserum to the peptide within two passages after being established. Staining was done on cultures that were maintained without dexamethasone or with 1 or 25 μM dexamethasone. No stain was seen in non-transfected Vero cells, Vero cells transfected with pMAM*neo*-BLUE or in Vero cells transfected with pDB339. Each of the Vero cell lines transfected with pDB337 showed positive staining when cultured in the presence of 1 or 25 μM dexamethasone but not in the absence of dexamethasone (Fig. 1). Staining was intense, restricted to the cytoplasm, and seen in almost 100% of the cells. The appearance was similar with each of the cell lines.

At passages 10–12 the immunoperoxidase staining was repeated, and the ELISA (Table 1) was performed on each Vero cell line. By this time only the H11.1 cell line expressed the peptide, as shown by both assays, in the presence of 1 or 25 μM dexamethasone, but not in the absence of dexamethasone. The other lines B-7, B-10, F-7 and F-9 had become peptide-negative at this time. The inducibility of peptide expression was also confirmed in H11.1 at passages 15–19 using various doses of dexamethasone (Table 2).

At passage 40 the H11.1 cell line was examined by the PCR to ensure that it still retained the viral sequences. A band corresponding to 410 bp was seen, and hybridised to the probe derived from the pDB337 DNA (not shown).

In the human keratinocyte cultures no non-transfected cells were stained with anti-T antibody, but in the cells transfected with pZ189 the proportion of stained cells was 48/4760 (1%). In the SV-T2 cell cultures, every cell showed nuclear staining.

Mutation frequency in cell lines

The mutation frequency of pK189 in Cos-1 cells was 0.024%, which is very similar to that obtained previously with pS189. Thus, the replacement of the ampicillin-resistance

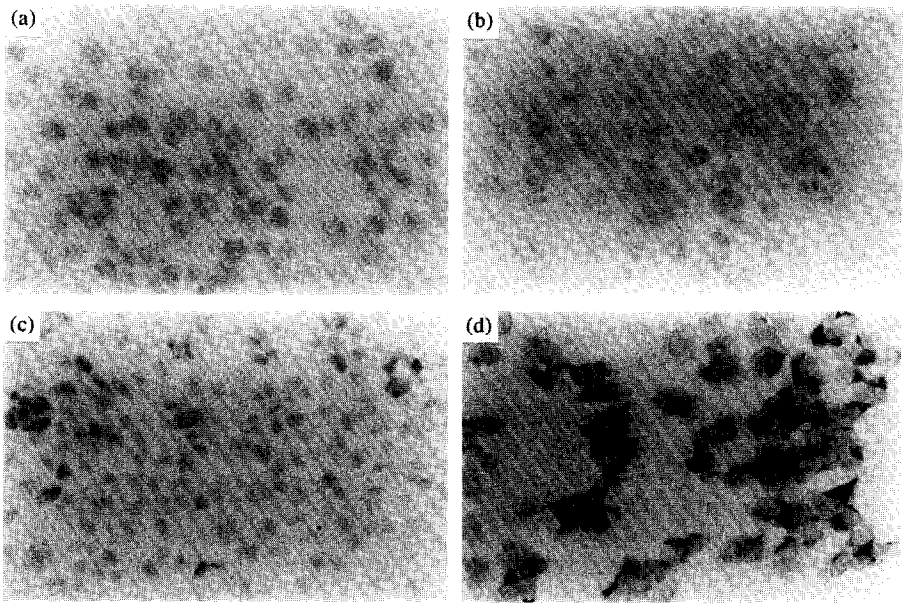

Fig. 1. Photomicrographs showing staining of different cell lines with antiserum to mutagenic peptide. (a) Vero; (b) Vero/pMAM*neo*-BLUE; (c) Vero/pDB339; (d) H11.1.

Table 1. *Induction of peptide in cell lines by dexamethasone as measured by an ELISA assay*

Cells	Concentration of dexamethasone		Increase
	None	25 μM	
Vero/pMAM*neo*-BLUE	0.463 (±0.014)*	0.445 (±0.067)	−0.02
Vero/pDB339	0.380 (±0.004)	0.373 (±0.011)	−0.01
H11.1	0.440 (±0.020)	0.949 (±0.014)	0.51
B7	0.464 (±0.060)	0.452 (±0.090)	−0.01
B10	0.511 (±0.060)	0.592 (±0.010)	0.08
F7	0.668 (±0.054)	0.519 (±0.120)	−0.15
F9	0.480 (±0.097)	0.418 (±0.020)	−0.06

*A_{414} values expressed as means ± standard error of triplicate readings. The assay was performed at passage 12.

Table 2. Induction of peptide in H11.1 cells by dexamethasone as measured by an ELISA assay

Dexamethasone	A_{414} values*	Increase†
None	0.150 (±0.04)	
1 μM	0.551 (±0.18)	5
5 μM	0.375 (±0.07)	4
25 μM	0.291 (±0.09)	3

*A_{414} values are expressed as means ± standard error of triplicate readings.
†Fold-increase was obtained in comparison with the A_{414} value obtained in the absence of dexamethasone. All A_{414} values were corrected by subtraction of the reading with pre-immune serum (0.486 ± 0.074). The assay was performed at passage 17.

gene with a kanamycin-resistance gene had no significant effect on the mutagenesis plasmid. In the presence of 25 μM dexamethasone the spontaneous mutation frequency in Vero cells was 0.21% which is higher than in Cos-1 cells. The highest mutation frequency was seen in the H11.1 cells, while the other peptide-expressing cells showed lower mutation frequencies (Table 3). However, these tests were done at relatively high passage levels, by which time some of the lines were no longer expressing peptide. In the H11.1 cells the mutation frequency increased with increasing concentrations of dexamethasone in the medium although other cells showed no change in the frequency with added dexamethasone (Table 4).

The types of mutations that occurred in the Vero and H11.1 cells were analysed by examination of digested fragments of the plasmid that were recovered from the cells. In no case had plasmid pDB337 been recovered in place of pK189. In Vero cells, point mutations accounted for 53% of mutations. Of the 47 plasmids with altered mobility, 37 showed slight reductions in size of one fragment and eight showed an increase in size of one fragment. Two plasmids were linearised by *Bam* HI and the single fragment was larger than 5.2 Kb; they were classified as insertions. In the H11.1 cells 54% of mutant plasmids were classified as having point mutations. Of the 46 with altered gel mobility, 39 showed a slight reduction in size of one fragment and one showed an increase in size of one fragment. Six plasmids showed neither simple increases nor decreases in one or the other fragments, but five of those were classified as insertions and one as a deletion based on the total size of all fragments. The types of mutant plasmids derived from each cell type are summarised in Table 5 and did not differ significantly between Vero and H11.1 cells.

Table 3. Frequency of mutations in cells lines in the presence of dexamethasone

	Colonies		Mutations	
Cells	White	Blue	Frequency*	*P*†
Vero	45	26 500	0.17%	n.s.
Vero/pMAM*neo*-BLUE	40	19 000	0.12%	n.s.
Vero/pDB339	34	11 800	0.29%	<0.05
H11.1	148	19 200	0.77%	<0.001
B7	21	12 400	0.17%	n.s.
B10	44	18 500	0.24%	n.s.
F7	59	19 800	0.30%	<0.01
F9	71	18 300	0.39%	<0.001

*Mutation frequency expressed as a percentage, derived from counting at least 15 000 colonies. The assay was performed at passages 10–12.
†The frequency of mutations was compared with that in the same cells in the absence of dexamethasone, using Fisher's exact test.
n.s.: not significant.

Table 4. Mutation frequency in cells in the presence of different concentrations of dexamethasone

	Concentration of dexamethasone				
Cells	None	1 μM	5 μM	10 μM	25 μM
Vero	0.21*	0.20	0.19	0.13	0.19
Vero/pMAM*neo*-BLUE	0.20	0.14	0.17	0.21	0.26
Vero/pDB339	0.12	0.11	0.10	0.11	0.29‡
H11.1	0.19	0.37†	0.40‡	0.62‡	0.76‡

*Mutation frequency expressed as a percentage, derived from counting at least 15 000 colonies. The assay was performed at passages 10–12.
†Significantly higher than mutation frequency of same cells in the absence of dexamethasone ($P < 0.01$).
‡Significantly higher using the same comparison ($P < 0.001$).

Table 5. Types of mutations observed in Vero and H11.1 cells

Type of mutation	Vero cells*	H11.1 cells
Point mutation	53	54
Deletion	37	40
Insertion	10	6

*One hundred mutant plasmids were examined from Vero cells and another 100 from H11.1 cells.

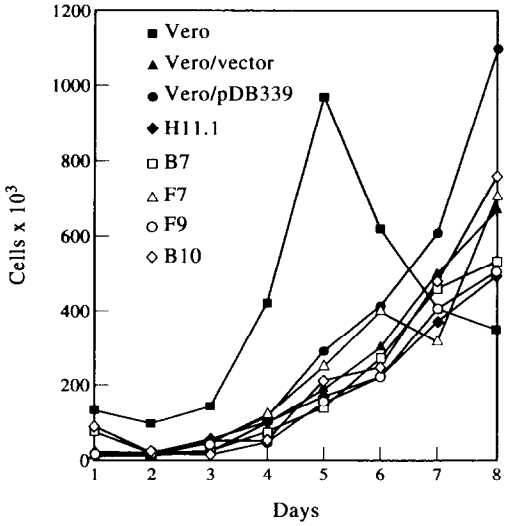

Fig. 2. Growth of cell lines. Cells were plated into wells of 24-well culture plates at a concentration of 1×10^4 cells per well in the presence of 25 μM dexamethasone. At daily intervals duplicate wells were harvested and cells counted.

Growth rate

Growth rates were measured for all cell lines that had been derived from Vero cells at pasage 8 or earlier and the results are shown in Fig. 2. At this passage, H11.1, B7, B10 and F9 expressed the peptide. F7 showed weak expression while Vero/pMAM*neo*-BLUE cells and Vero/pDB339 cells did not express the peptide as determined by immunostaining.

Non-transfected Vero cells grew rapidly when plated at a concentration of 1×10^4 cells per well and reached their peak number on day 5, stayed on a plateau for a few days and then the number of cells declined rapidly. Each of the transfected cell lines grew more slowly, apparently since a smaller number of them became plated. They were very similar to each other up to day 4. However, from day 5 onwards the peptide-positive lines H11.1, B7, B10 and F9 grew at only half the rate of the peptide-negative Vero/pDB339 line. All these lines reached peak densities on day 8 or later. The growth rate of F7 followed the pattern close to the Vero/pDB339 line. Growth curves were measured three times using each cell line, with similar results.

Serum dependence

When cells were plated with different concentrations of serum, two distinct patterns of survival were seen. The Vero/pMAM*neo*-BLUE cells, the Vero/pDB339 cells and the B10 cells showed increasing numbers of foci with increasing concentrations of serum. However, the H11.1 and B7 cells showed a maximum number of foci with 0.6% serum, and fewer foci with either more or less serum (Fig. 3). The F9 and F7 cells showed a pattern most consistent with the Vero/pMAM*neo*-BLUE cells, although with fewer foci at each concentration of serum. H11.1 cells formed colonies that were larger in size than any other lines, and could form foci at a lower serum concentration, 0.2%, than any other line (Fig. 4).

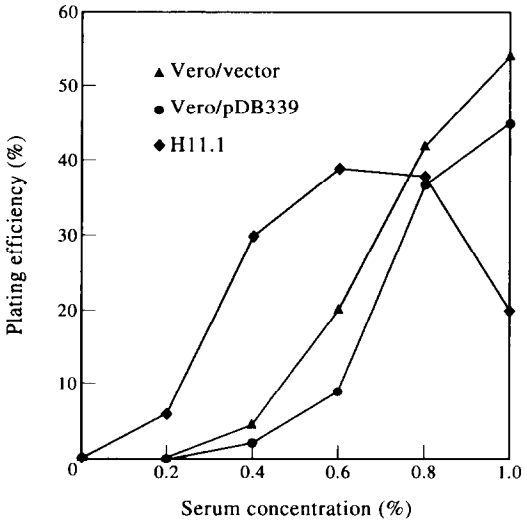

Fig. 3. Serum dependence of cell lines. Cells at the 10th to 12th passage were plated in 100 mm dishes in media containing different concentrations of serum. Growing colonies were counted after 12 days and expressed as a percentage of cells that had been plated.

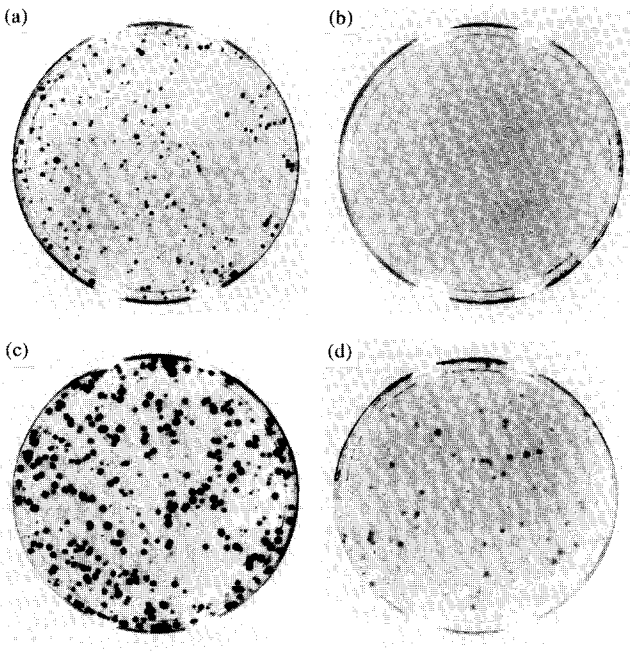

Fig. 4. Appearance of cell colonies from experiments shown in Fig. 3. (a) Vero/pDB339 cells, 10% serum; (b) Vero/pDB339 cells, 0.5% serum; (c) H11.1 cells, 10% serum; (d) H11.1 cells, 0.5% serum.

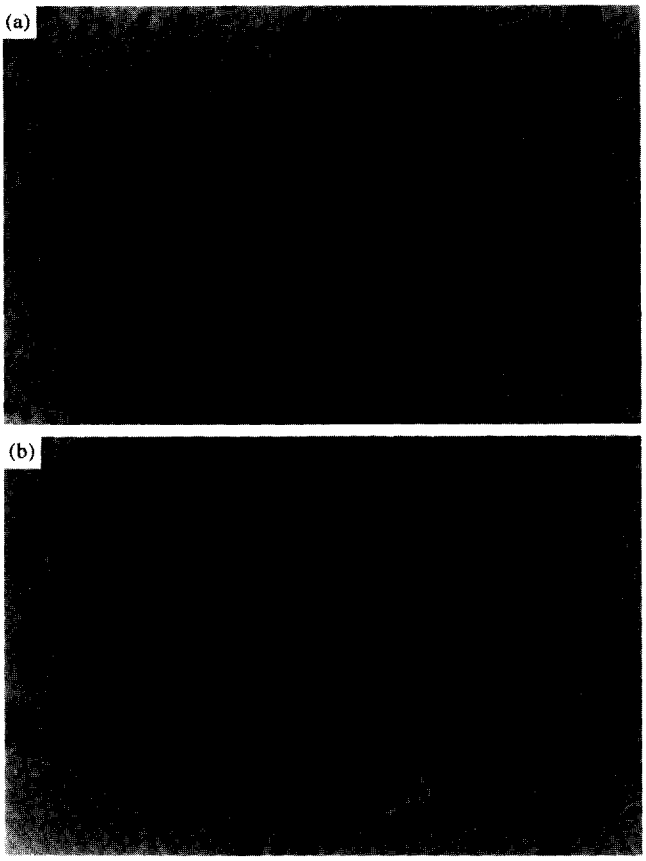

Fig. 5. Anchorage independence of transfected cells. Cells were seeded in methylcellulose and anchorage-independent colonies were photographed after 21 days. HeLa cells were included for comparison. No colonies were seen with cells that were non-transfected, or were transfected with pDB339. (a) HeLa cells; (b) H11.1 cells.

Anchorage independence

None of the transfected Vero cell lines could make anchorage-independent colonies at early passages. However, after the H11.1 cells had reached passage 30 they were capable of growing as anchorage-independent foci, producing the same number of foci per dish as HeLa cells (200 colonies/10^5 cells), although each focus was about half the diameter (Fig. 5). Vero cells that were maintained in parallel cultures for the same time did not develop the ability to form foci.

Transformation of human cells

All cultures survived transfection with each plasmid and continued to grow. Monolayer diameters were consistently smaller, by 15–20% than those of non-transfected cultures, but no differences were seen between cultures that were transfected with different plasmids. All cultures were terminally differentiated by 22 days and died. Transformation experiments with the human cells were performed five times, using primary cultures from five different cell donors, with similar results.

Tumorigenicity

No tumours had appeared by 3 months in animals that received any dose of transformed NRK536 cells, Vero cells or H11.1 cells. No tumour foci were detected by histological examination of the injection sites. However, animals that received the control, tumorigenic cell line SV-T2, at a dose of 1×10^7 cells, all developed tumours with an average diameter of 1.5×0.7 cm by the 6th week.

DISCUSSION

It is clear that HSV-1 can transform cells to a malignant phenotype since the phenomenon has been demonstrated repeatedly [1, 4]. The basis of this remains obscure, but the lack of viral DNA in the transformed cells implies that the virus acts as a mutagen. The transforming region of the genome has recently been shown to encode a peptide which raises the mutation frequency of cells, and patients with oral cancer have serum antibody against the peptide [18]. Since mutagenesis is a characteristic of many carcinogens, it might be anticipated that the peptide would transform cells to a malignant phenotype.

As expected, the mutagenic peptide did lead to morphological transformation of NRK536 cells. These cells were also readily transformed by HSV-1 particles, as reported by others [11], and the plasmid pDB337 was as efficient as the virus particle in producing foci. However, the cells were not tumorigenic in nude mice, indicating that the peptide had not induced complete transformation.

When Vero cells were transfected with the plasmid that expressed the mutagenic peptide they were slower to show features of transformation. This was despite the fact that in each of the five lines examined the expression of the peptide could be induced at early passages and the mutation frequency was raised (Table 3). Four lines lost the ability to express the peptide by passage 10, but the H11.1 line continued to express the peptide and retained the viral sequences up to at least passage 40. The H11.1 cells were able to grow in a lower serum concentration than the non-transfected cells when expression of the peptide was induced. The cells did not form foci in soft agar or induce tumours in nude mice, indicating that the peptide had only induced some of the features of transformation.

Human oral cells showed no phenotypic changes that could be attributed to the viral peptide. This was despite controls showing that transfection was achieved successfully. In other experiments we found that the lifespan of human oral keratinocytes can be extended by N-nitrosamines [19]. Therefore the cells were susceptible to phenotypic modifications, but the viral peptide was not capable of inducing such changes.

The results of this study show that the mutagenic peptide of HSV-1 could be the viral protein that is responsible for cell transformation to an immortal phenotype or to the anchorage-independent phenotype that has been reported in other experiments. However, transformation to a malignant phenotype must require other viral proteins that are also encoded within *mtr*-1. The data suggest that transformation of human cells cannot be attributed to this viral peptide alone.

The activities of this virus peptide that might be responsible for changing the cell phenotype are unknown. However, it is known that the mutagenic peptide represents the carboxy terminus of one member of a family of virus-encoded proteins. These proteins have activities that include virion assembly [20], protease and substrate activities [21, 22], and kinase and DNA-binding properties [5]. Any of these functions might be involved in cell transformation. The present study confirms that the peptide is antigenic, and its role in oral cancer has been suggested by the observation that patients with oral cancer

have increased levels of IgM antibody to the mutagenic peptide. This suggests that it is expressed at some point in the development of the tumour [18]. The peptide, therefore, might play several different roles during viral infection, as do most of the transforming proteins of DNA viruses [23].

It is now important to test the effects of the mutagenic peptide on oral cells that have been immortalised, or partially transformed in other ways. Oral cells that have been immortalised by papillomaviruses can become fully transformed by subsequent exposure to an alkylating chemical carcinogen [24], and so might also be transformed by the HSV-1 peptide. Alternatively, our oral cells whose survival was extended by exposure to tobacco-related carcinogens [19] might be susceptible to transformation by the peptide. These studies are in progress.

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Acknowledgements—The authors wish to thank Dr M.P. Moyer for helpful advice with the human cell cultures. This study was supported by PHS grant DE07007. C.D. was the recipient of NRS award F32 DE05577. The synthetic peptide was made in the Synthetic Antigen Laboratory, M.D. Anderson Cancer Center, which is supported by PHS grant CA 16672.